A Transglutaminase-Related Antigen Associates with Keratin Filaments in Some Mouse Epidermal Cells

Sophie Clement,[1] Amy V. Trejo-Skalli,[1] Lin Gu, Pauline T. Velasco, Laszlo Lorand, and Robert D. Goldman

Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, U.S.A.

A mouse monoclonal IgG, G82, directed against guinea pig liver transglutaminase recognizes a transglutaminase-related antigen that is associated with the keratin intermediate filament network in some primary mouse keratinocytes. The association can be seen at the resolution of individual keratin tonofibrils following fixation and staining for double-label indirect immunofluorescence. Western blots indicate that G82 reacts with two proteins of 95 kDa and 280 kDa, respectively, in extracts of these cells. The 95-kDa band is also recognized by a polyclonal antibody against purified guinea pig liver transglutaminase, and the 280-kDa protein seems to correspond to a similar protein that was shown to be recognized by G92.1.2 in the intermediate filament fraction of primary mouse fibroblasts. The transglutaminase-related antigen was shown by confocal microscopy to co-localize only with nonbasal cell specific keratin intermediate filaments. ***Key words: differentiation/intermediate filaments/keratinocyte. J Invest Dermatol 109:778–782, 1997***

Transglutaminases (TGase) are post-translationally acting enzymes that are widely distributed in many cell types. New forms in this family of enzymes are being reported with increasing frequency. They function by modifying the γ-amides of specific glutaminyl residues in their protein substrates either by hydrolysis or by transamidation (Folk, 1980; Lorand and Conrad, 1984). Their activities often lead to the formation of polymeric protein products cross-linked by Nε-(γ-glutamyl)lysine bridges. This type of cross-linking, especially of membrane-associated and cytoskeletal proteins [as seen in human erythrocytes (Siefring *et al*, 1978) or keratinocytes (Rice and Green, 1979)], may cause irreversible alterations in cell shape with loss of cytoskeletal plasticity (Smith *et al*, 1981).

An influx of Ca^{++}-ions may serve as the immediate trigger for activating latent TGase present in cells (Siefring *et al*, 1978); however, stimulation of cells by other means may also produce the same effect, perhaps mediated through an increase in the intracellular concentration of Ca^{++}. This has been shown to be the case following the interaction of thrombin with human platelets (Lorand *et al*, 1987), the fertilization of the sea urchin egg by the sperm (Cariello *et al*, 1984, 1990), and the stimulation of human epidermal carcinoma A431 cells by epidermal growth factor (Dadabay and Pike, 1987). Increases in the potencies of TGase have been associated with several other cellular activities including changes in morphology and adhesiveness (Gentile *et al*, 1992), growth inhibition (Dell'Orco *et al*, 1985), differentiation (Kannagi *et al*, 1982), apoptosis (Fesus *et al*, 1987), and G protein activity (Nakaoka *et al*, 1994).

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Reprint requests to: Laszlo Lorand, Department of Cell and Molecular Biology Northwestern University Medical School, Searle Building 4–555, 303 East Chicago Avenue, Chicago, IL 60611.

Abbreviations: IF, intermediate filament; TGase, transglutaminase; TRA, transglutaminase-related antigen.

[1]Both authors contributed equally to this work.

We have found that a transglutaminase-related antigen (TRA), identified by a monoclonal antibody (MoAb) directed against guinea pig liver TGase, is associated with the vimentin-containing intermediate filaments (IF) of primary mouse skin fibroblasts (Trejo-Skalli *et al*, 1995). As an extension of these studies, we now report that some keratinocytes in primary mouse skin cultures also contain a TRA associated with the keratin IF network.

MATERIALS AND METHODS

Primary mouse epidermal cell cultures Keratinocytes were isolated from 36-h BALB/C mice by the method of Yuspa and Harris (1974). Cell growth was promoted by culturing in minimum Eagle's medium (Gibco BRL, Grand Island, NY) without calcium, containing 10% calcium-chelated (Chelex-100, Bio-Rad, Hercules, CA) fetal bovine serum (0.02 mM Ca^{++}). Differentiation was induced by switching to minimum Eagle's medium containing 1.4 mM Ca^{++} and 10% fetal bovine serum for either 4 or 24 h.

Antibodies Purified guinea pig liver TGase (Croall and DeMartino, 1986; Jeong *et al*, 1995) was used as an antigen to prepare both a rabbit polyclonal anti-serum and the mouse MoAb, G82. The latter was prepared from a subclone of the previously described mouse MoAb G92 (Trejo-Skalli *et al*, 1995), and grown in a bioreactor for 80 d. IgG was purified from culture medium using a GammaBind Plus Sepharose column (Pharmacia LKB, Piscataway, NJ).

Rabbit polyclonal antibody against bovine tongue keratin was prepared according to the procedure of Jones *et al* (1988). Polyclonal antibodies directed against keratin 5 were produced using the specific C-terminal peptide KYTTTSSSKKSYRQ as antigen. These were purified by affinity chromatography with the peptide covalently linked to Affigel 10 (Bio-Rad) beads in accordance with the manufacturer's instructions (10 mg of peptide coupled to 10 ml of beads). One ml fractions were eluted from the column with 100 mM glycine, pH 2.5 and neutralized with 0.1 ml of 1 M Tris-HCl, pH 8.2, and those containing antibodies were pooled and dialyzed overnight against phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.154 M NaCl, pH 7.4).

Enzyme-linked immunosorbent assay (ELISA) Purified guinea pig liver TGase (Croall and DeMartino, 1986), human red blood cell TGase (Radek *et al*, 1993), chicken red blood cell TGase (Weraarchakul-Boonmark *et al*, 1992), and recombinant human factor XIII A subunits (Bishop *et al*, 1990; we gratefully acknowledge gift of this protein from Dr. Paul Bishop, Zymogenetics, Seattle,WA) were plated at 0.2 μg per well for 2 h in a microtest plate (Falcon,

Lincoln Park, NJ). Nonspecific binding sites were blocked for 30 min with 2% bovine serum albumin (Sigma, St Louis, MO) in PBS. Subsquently, MoAb G82 was added to each well for 2 h, followed by a wash with blocking solution and by the addition of an alkaline phosphatase-conjugated antibody directed against mouse IgG (Sigma, diluted 1:5000 in blocking solution). Wells were washed with 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, and p-nitrophenyl phosphate disodium (Sigma; 1 mg per ml in the above buffer) was added to each well. After 30 min, color was read in a Dynatech MR600 microplate reader, at 410 nm.

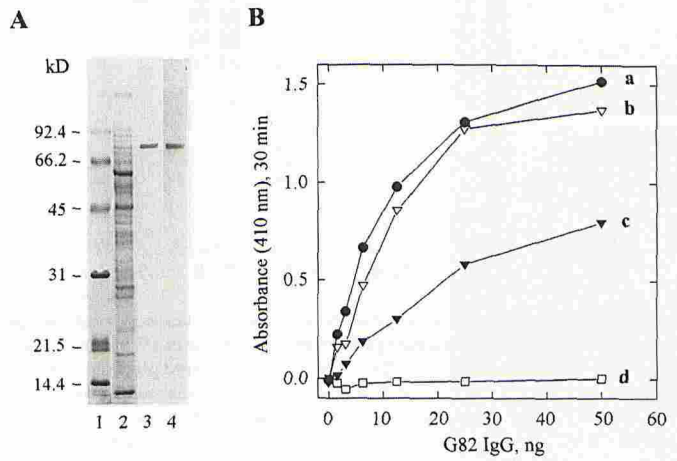

Figure 1. Binding of antibody G82 to different types of TGase. (*A*) Immunoblots of guinea pig liver homogenate with two different TGase antibodies. Guinea pig liver homogenate was subjected to SDS-PAGE in a 10% acrylamide gel (30 μg per lane) and transfered to nitrocellulose. *Lane 1*, molecular weight markers stained with amido black and $M_r \times 10^{-3}$ values as indicated; *lane 2*, liver homogenate stained with amido black; *lanes 3* and *4*, immunoblots of the liver homogenate with a polyclonal serum and the monoclonal G82 IgG against guinea pig liver TGase, respectively. (*B*) Purified TGase from guinea pig liver (*curve a*), human red blood cells (*curve b*), chicken red blood cells (*curve c*), and also the A subunits of recombinant factor XIII (*curve d*) were plated and the binding of antibody G82 was measured by the ELISA procedure.

Protein extracts, gel electrophoresis, and immunoblot analysis Whole cell extracts and IF-enriched cell extracts (Starger *et al*, 1978) were obtained from primary cultures of keratinocytes induced to undergo differentiation for 4 h. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel, Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al*, 1979). Blots were stained for protein with 1% amido black (Sigma) in 15% acetic acid, 50% methanol. For immunoblotting, primary antibodies were diluted in a 2% milk/PBS solution as follows: rabbit anti-serum directed against bovine tongue keratins, 1:5000; rabbit anti-serum against mouse keratin 5, 1:2000; TGase anti-serum, 1:5000; and the MoAb G82 was used at a final concentration of 0.3 μg per ml. Treatments with secondary antibody were carried out with either alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega, Madison, WI; 40 μg per ml) or peroxidase-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA) or anti-mouse (Kirkegaard and Perry, Gaithersburg, MD) antibodies (40 μg per ml). Alkaline phosphatase activity was determined using 5-bromo-4-chloro-indolyl phosphate (Sigma, 300 μg per ml) as substrate and nitro blue tetrazolium (Sigma, 150 μg per ml) as electron acceptor in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. Peroxidase activity was detected by chemilluminescence employing an ECL kit (Amersham, Arlington Heights, IL).

Guinea pig liver homogenate (Blobel and Potter, 1966) was subjected to SDS-PAGE in 10% gel (30 μg per lane) and transferred to nitrocellulose. Immunoblot analysis was carried out with the guinea pig liver TGase anti-serum diluted 1:5000 or with G82 (0.13 μg per ml). Following incubation overnight, alkaline phosphatase-conjugated goat anti-rabbit (Promega) or anti-mouse (Sigma) antibodies were used at a dilution of 1:5000. Alkaline phosphatase activity was measured as described above.

Immunofluorescence Mouse epidermal keratinocytes grown and induced to undergo differentiation on glass coverslips were fixed in ice-cold methanol as recommended by Yang *et al* (1985). For double-label indirect immunofluorescence, 100 μl of G82 (85 μg per ml in PBS) was applied to coverslips, followed by incubation with fluorescein-conjugated goat anti-mouse antibody (Kirkegaard and Perry, 25 μg per ml in PBS). Subsequently, the cells were stained either with the polyclonal rabbit antibody directed against bovine tongue keratin (1:20 dilution) or with the affinity purified polyclonal keratin 5 antibody (1:20 dilution). This was followed by incubation with rhodamine-conjugated goat anti-rabbit secondary antibody (Kirkegaard and Perry, 25 μg per ml). Cells were observed with either a Zeiss Axiophot or a Zeiss Laser Scan 410 confocal microscope (Germany). Micrographs were taken on 35 mm film or stored on optical disks.

RESULTS AND DISCUSSION

Antibody G82 is specific for several types of TGase Binding of the MoAb G82 to various TGase was tested by ELISA, as well as

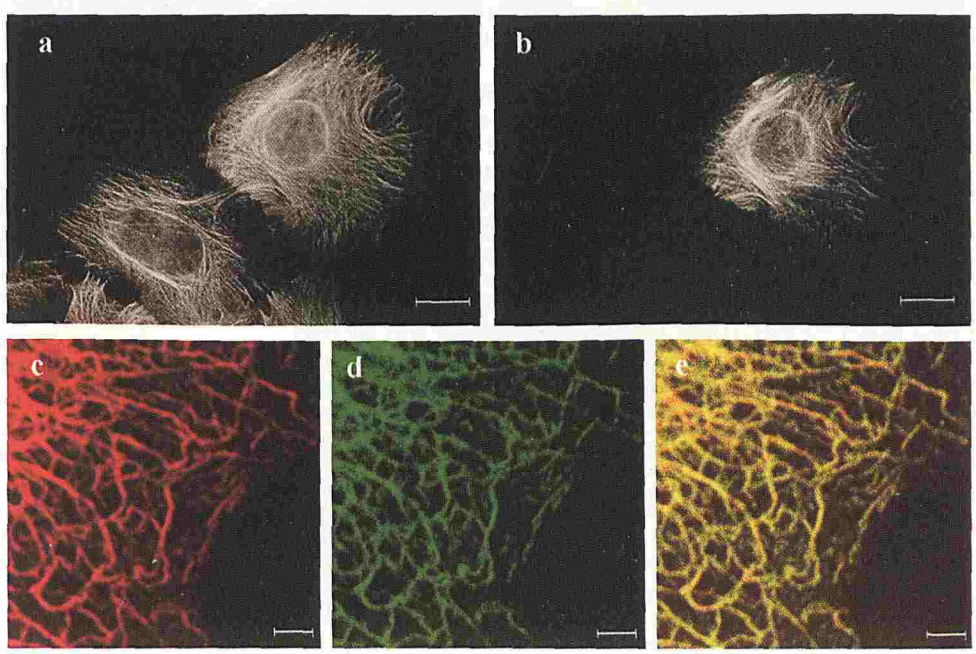

Figure 2. Double-label indirect immunofluorescence of epidermal keratinocytes. Anti-keratin (*a*, *c*) and anti-TGase G82 (*b*, *d*). Confocal microscopy was used to demonstrate the precision of co-localization at high magnification (*c–e*). Overlay of images (*c*) and (*d*) is shown in (*e*). *Scale bars*, (*a*, *b*) 15 μm, (*c–e*) 5 μm.

by immunoblotting. **Figure 1*A*** reveals that G82 reacts with an 80-kDa protein in the homogenate of guinea pig liver (*lane 3*) that is also recognized by the polyclonal anti-serum directed against TGase (*lane 4*). ELISA experiments with purified TGase indicate that, in addition to recognizing purified guinea pig liver enzyme, G82 also reacts with the human red blood cell TGase, and to a lesser extent with the enzyme isolated from chicken red blood cells (**Fig 1*B***). It is interesting to note that the antibody recognizes these antigens only in the immobilized form, attached to nitrocellulose or to microtiter plates, and it does not bind to the purified transglutaminases in solution [i.e., mixing the enzymes with MoAb G82 in solution does not reduce its ability to bind to the plated antigen in ELISA competitive assays (data not shown)]. Thus, we conclude that MoAb G82 recognizes a conformationally sensitive epitope in transglutaminase.

A TGase-related antigen co-localizes with keratin IF in mouse epidermal keratinocytes We previously found that mouse monoclonal IgG, G92.1.2, raised against guinea pig liver TGase, recognizes a TRA closely associated with vimentin IF networks in mouse primary dermal fibroblasts (Trejo-Skalli *et al*, 1995). This antibody did not stain the epidermal keratinocytes; however, we now find that another MoAb (G82), also directed against guinea pig liver TGase, recognizes a TRA that co-localizes with keratin IF in some of the keratinocytes in primary cultures of mouse epidermal cells. MoAb G82 also reacts with the TRA found in association with the vimentin IF in dermal fibroblasts (data not shown).

Mouse keratinocytes, induced to undergo differentiation for 4 h, were processed for double-label immunofluorescence using MoAb G82 and an anti-serum directed against bovine tongue keratins (**Fig 2**). In western immunoblot assays, the latter anti-serum reacts with all keratins present in mouse epidermal keratinocytes (Jones *et al*, 1988; **Fig 3**, *lanes 7* and *8*). The results show that the MoAb G82 recognizes a TRA present only in a subset of keratinocytes (**Fig 2*b***). The number of G82 positive cells varies from 15 to 30% among different cultures. This may indicate a regulated expression of the TRA and the variations in the percentage of cells expressing the antigen may be a reflection of minor differences in the states of differentiation of cells in the primary keratinocyte cultures. In those cells that stain with G82, however, there is always a co-localization with keratin IF (**Fig 2*a,b***). The high degree of coincidence between the two antigens could be readily demonstrated at the level of resolution of individual keratin tonofibrils by confocal microscopy (**Fig 2*c–e***). In some cases, the TRA appears to be dispersed in a punctate pattern along the keratin tonofibrils.

The TGase-related antigen co-isolates with keratin in the IF-enriched cell fractions Whole cell extracts and keratin IF-enriched cell fractions from primary mouse keratinocytes induced to differentiate for 4 h were analyzed by western blotting. In whole cell extracts (**Fig 3**), G82 reacts with both a 95-kDa and a 280-kDa protein (*lane 3*). *Lanes 7* and *8* show that G82 does not cross-react with the keratins present within the keratinocytes. In IF-enriched fractions, only the 280-kDa component was detected by the G82 MoAb. The 280-kDa TRA corresponds to a protein first detected by its reaction with the related MoAb G92.1.2 in the IF-enriched fraction of primary mouse fibroblasts (Trejo-Skalli *et al*, 1995). The 95-kDa protein that is recognized by G82 in whole cell extracts was identified as a cytosolic TGase because of its cross-reaction with the polyclonal antibody raised against guinea pig liver TGase. The polyclonal antibody does not react with the 280-kDa species (*lanes 5* and *6*), and, instead of giving a filamentous staining pattern in the primary keratinocyte culture, presents a diffuse cytoplasmic distribution (data not shown). These findings indicate that only the 280-kDa TRA is associated with the keratin IF network.

The keratin IF-associated TGase-related antigen is present only in differentiated keratinocytes After 24 h of exposure to 1.4 mM Ca^{++}, keratinocytes in culture form islands comprised of differentiated cells in the center and in the upper layers, and with the undifferentiated cells at the periphery (Hennings *et al*, 1980). This was confirmed by staining with an antibody against basal cell keratin 5 that recognizes

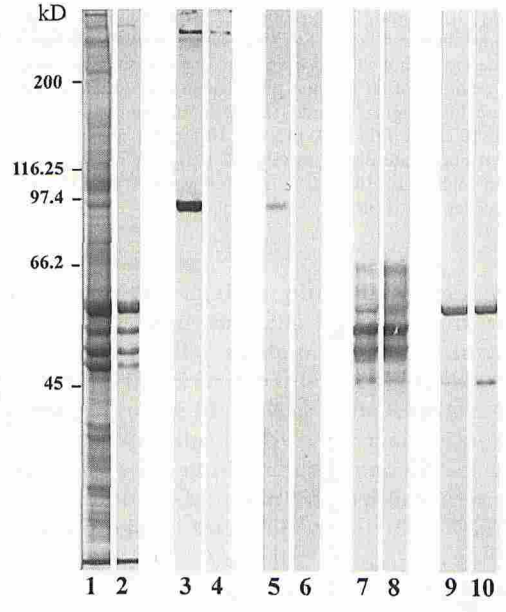

Figure 3. Western blots show the presence of a TGase-related antigen in extracts of primary mouse epidermal keratinocytes. Whole cell extract (*odd lanes*) and an IF-enriched cell fraction (*even lanes*) were prepared from cells induced to undergo differentiation for 4 h. Extracts were subjected to SDS-PAGE in a 10% acrylamide gel followed by western blotting with either TGase MoAb G82 (*lanes 3* and *4*), anti-serum to guinea pig liver TGase (*lanes 5* and *6*), anti-serum to bovine tongue keratins (*lanes 7* and *8*), or anti-serum to keratin 5 (*lanes 9* and *10*). *Lanes 1* and *2* were stained with amido black.

primarily one of the keratins present in these cells (see *lanes 9* and *10* in **Fig 3**). Using confocal microscopy, the keratin 5 antibody stains only peripheral cells, whereas the bovine tongue keratin anti-serum recognizes the keratins present in all keratinocytes of the cell islands (**Fig 4**). Double-label immunofluorescence shows that the TRA reacting with the G82 antibody is present only in the centrally located, keratin 5 negative cells. These observations suggest that TRA association with the keratin network occurs exclusively in the terminally differentiating keratinocytes.

The functional significance of the association between TRA and keratin IF remains to be determined; however, keratin is a known substrate of the ≈90 kDa form of TGase in keratinocytes of skin (Steinert and Idler, 1979). The possibility exists that the TRA represents a high molecular weight precursor form of TGase, a TGase covalently attached to keratin filaments by some bonds other than disulfides or a novel TGase that is coexpressed with keratin IF during the course of keratinocyte differentiation. The association of these two proteins may ultimately lead to a greater insight into the role of TGase in the terminal differentiation process that takes place in cells of the stratum corneum. Furthermore, these observations may also provide clues with regard to the mechanisms underlying the regulation of IF stability. It has recently been shown that IF play an important role in maintaining cell shape and that their dynamic nature may allow for shape changes to occur (Coulombe *et al*, 1991; Miller *et al*, 1991, 1993; Goldman *et al*, 1996). Maintenance of cell shape and preservation of tissue architecture are critical for the formation of a complex organ such as skin, and some loss in the dynamic nature of IF might be required to achieve a certain degree of stability in the structure of stratum corneum. Cross-linking by an enzyme such as TGase could conceivably provide this stability by lowering the rate of exchange between the cytoplasmic pools and filamentous forms of IF (Vikstrom *et al*, 1992).

Another possible function of the TRA might be to serve as an IF-aggregating protein playing a role in the bundling of individual keratin filaments during keratinocyte differentiation. A similar function in the formation of these tonofibrils has been attributed to filaggrin, another keratin IF-associated protein (Dale *et al*, 1978).

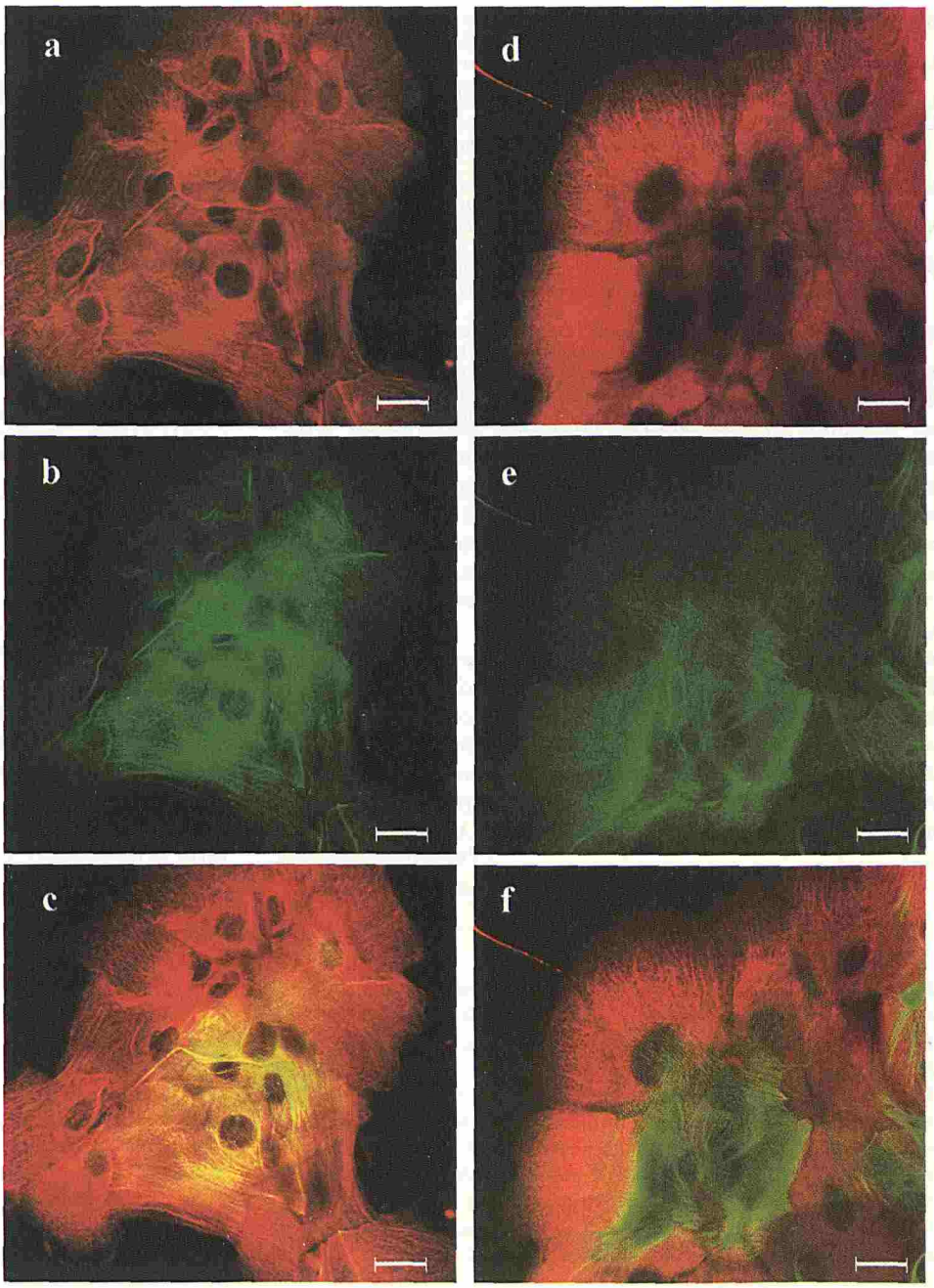

Figure 4. Double-label indirect immunofluorescence of 24-h differentiated keratinocytes. TGase antibody G82 (*b*, *e*) and two polyclonal antibodies directed against keratin, anti-bovine tongue keratin (*a*) that recognizes all keratins in cultured keratinocytes, and anti-keratin 5 (*d*) that recognizes the basal cell specific keratin, were used for staining. Overlay of images (*a*, *b*) and (*d*, *e*) are shown in (*c*) and (*f*), respectively. *Scale bars*, 25 μm.

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